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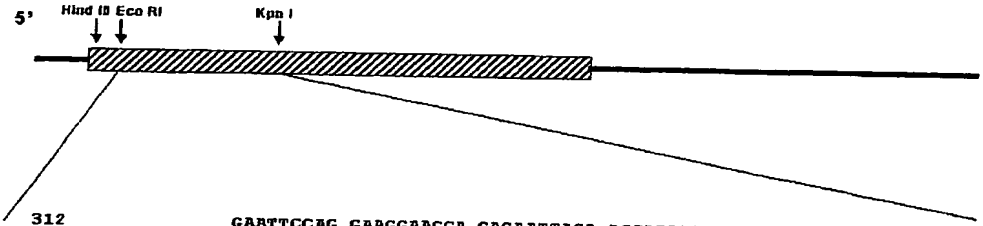
(54) Title: PROCESS FOR THE DETERMINATION OF THE PRIMARY STRUCTURE OF THE MESSENGER RNA CODING FOR THE HUMAN RECOMBINANT ENDOOLIGOPEPTIDASE A (HEOPA) [AF217798]...

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312                GARTTCCAG GAAGGAAGCA GAGAATTAGA AGCAGAGTTG GAGGCACAAT
361  TAGTACAGGC TGAACAAAGA AATAGAGACT TGCAGGCTGA TAACCAAAGA CTGAAATATG
421  AAGTGGAGGC ATTAAAGGAG AAGCTAGAGC ATCAATATGC ACAGAGCTAT AAGCAGGTCT
481  CAGTGTTAGA AGATGATTTA AGTCAGACTC GGGCCATTAA GGACCAGTTG CATAAGTATG
541  TGAGAGAGCT GGAGCAGGCC AACGACGACC TGGAGCGAGC CAAAAGGGCA ACAATAGTTT
601  CACTGGAAGA CTTTGAACAA AGGCTAAACC AGGCCATTGA ACGAAATGCA TTTTTAGAAA
661  GTGAACTTGA TGAAAAGGAA TCTTTGTTGG TCTCTGTACA GAGGTTAAAG GATGAAGCAA
721  GAGATTTAAG GCAAGAACTA GCAGTTCGGG AAAGACAACA GGAAGTAACT AGAAAGTCGG
781  CTCCTAGCTC TCCAACTCTA GACTGTGAAA AGATGGACTC CGCCGTACAA GCATCACTTT
841  CTTTGCCAGC TACCCCTGTT GGCAAAGGAA CGGAGAACAC TTTTCCTTCA CCGAAAGCTA
901  TACCAAATGG TTTTGGTACC
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(57) Abstract: This invention refers to the recombinant human endooligopeptidase (hEOPA), polynucleotide which codes for the hEOPA, polynucleotide which allow the expression of the EOPA in prokaryotes and eukaryotes, including the human beings; use of the synthetic substrates for the determination of the proteolytic activity of the hEOPA, or of its chaperon activity or its activity as soluble peptide receptor; obtaining and using of the specific antibodies and inhibitors of its oligopeptide binding activity, as agonists, competitors and antagonists, which are able to disturb its interaction and the complex formation with other proteins. The invention also refers to the application of the natural and recombinant protein, chemically or genetically modified which aim is the diagnosis and/or the application in congenital, infectious and degenerative pathologic conditions of the central nervous system, and for psychiatric and behavioral dysfunctions. It is also proposed the application of the inhibitors and competitors for the interaction of EOPA with ligands, including antibodies or their derivatives, for the treatment of tissular and neurodegenerative pathologies.

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Process for the determination of the primary structure of the messenger RNA coding for the human recombinant Endooligopeptidase A (*h*EOPA) [AF217798], and the respective protein sequence [AAF24516]; determination of the human EOPA gene and the production of the human recombinant EOPA; process of generating polyclonal anti-EOPA antibodies in mice; standardization and utilization of synthetic substrates to the characterization of the biochemical and proteolytic properties of *h*EOPA; process of identification and production of inhibitors and antibodies displaying inhibitory activity against either the EOPA catalytic activity or the EOPA ability to interact with oligopeptides acting as a "soluble receptor"; methods of identification of this protein in congenital, infectious and degenerative pathologies of the central nervous system, and methods of determination of EOPA's roles in immunological processes; immunochemical and/or enzymatic diagnosis methods to be employed for the prevention, accompanying the evolution of pathologies, prognosis or the treatment of congenital, infectious and/or degenerative diseases of the central nervous system; use of the interaction properties of ligands with the active center of EOPA, acting either as inhibitors of its enzymatic action and/or interfering with its association with other intracellular proteins, which can be useful for the treatment of neurological, psychiatric and degenerative pathologies.

The invention relates to the human recombinant endooligopeptidase A (*h*EOPA), to the polynucleotides coding for *h*EOPA, which allow the expression of the *h*EOPA in prokaryotes and eukaryotes, including in human beings; to the utilization of the synthetic substrates for the determination of the proteolytic activity of *h*EOPA, or for

the evaluation of its activity as chaperone or as peptides "soluble receptor"; to the obtaining and utilization of specific antibodies and inhibitors of its oligopeptide-ligand activity that can act as agonists, competitors and antagonists, able to influence on its interaction with other proteins and on the formation of multiprotein complexes. The invention also relates to the application of this protein, in its native, recombinant, chemically or genetically modified forms, for diagnosis and/or application in congenital, infectious and degenerative pathologies of the central nervous system, and for psychiatric, cognitive, and behavioral dysfunctions. It also proposes the application of inhibitors and competitors of the interaction of ligands to the EOPA, including antibodies and their derivatives for the treatment of neurological pathologies and tissue degenerations.

More specifically, the present invention relates to a protein expressed in the central nervous system (CNS), which shows proteolytic activity against peptides substrates, and displays a protein structure that allows its interaction with oligopeptides and/or intracellular proteins, determining important physiological functions like neuronal cell migration, neuritogenesis, interneuronal signaling (e.g., signaling for synapses formation), antigen presentation, and biotransformation and/or protection of bioactive peptides (chaperone activity), or modulating protein interactions of bioactive peptides ("soluble receptor" activity).

In order to reach the objectives of the present invention, a specific methodology is proposed to determine the primary structure of the messenger RNA coding for this

protein and, therefore, also the primary structure of the protein sequence of the human EOPA.

Likewise, the present invention provides the determination of the primary structure of the EOPA gene, and also of the regulatory factors controlling its transcription and expression.

In addition, the primary specificity of the EOPA was characterized, regarding to the substrate hydrolysis (proteolytic activity) and also to the interaction with peptides in its active center (chaperone or "soluble receptor"-like activity), thereby influencing the formation of active complexes, able to act in cellular processes of the central nervous system, and generating active products. It also relates to the generation and/or application of synthetic or natural specific inhibitors, which might be used "in vitro", "ex vivo", or "in vivo", influencing EOPA's physiological or pathological role.

As a further object of the present invention, it is described the generation of specific antibodies, which are able to both inhibit the catalytic activity of EOPA and to recognize EOPA specifically in tissues and biological fluids.

Another object of this invention is the use of either the polynucleotide encoding for the EOPA, the respective recombinant protein or the antibody generated against it for the determination of the distribution pattern of this protein in different tissues, organs and fluids of humans and animals.

As additional objectives, this invention also provides the determination of the expression and secretion of this protein in tumor tissues or cells; in totipotent (stem cells) and neuronal cells, during differentiation process,

formation of neurites or in the process of synaptic connection formation; and also in pathological processes, degenerative or repair thereof, especially of the nervous tissue and smooth muscle.

The several objectives of the present invention were achieved and characterized by diverse proceedings described as follows.

Cloning of the cDNA coding for the human EOPA and analysis of its deduced amino acid sequence

The cDNA coding for the human EOPA was isolated from a human brain cortex cDNA library, purchased from Stratagene (La Jolla, USA), after screening by hybridizations employing radioactive-labeled probes derived from a cDNA sequence coding for the rabbit EOPA, identified and described by the authors of the present invention (Hayashi et al., 2000), and deposited at the GenBank databank under the Acc. No. AF015037 and AAB99905, for the cDNA and the corresponding protein, respectively.

This approximately 2.2 kb long cDNA was completely sequenced, and the sequence was deposited at GenBank under the Acc. No. AF217798 and AAF24516, for the cDNA and the protein sequences, respectively.

The following steps were undertaken to determine the primary structure of the messenger RNA coding for the mammalian EOPA and the corresponding protein sequence:

a) total RNA was isolated from several animal tissues, employing the guanidine isothiocyanide-phenol-chloroform extraction method or, alternatively, using Trizol™ (GibcoBRL);

b) messenger RNA was purified from total RNA using pre-packed oligo-dT cellulose columns or the resin in suspension;

c) the quality of the resulting messenger RNA was analyzed by electrophoresis in denaturing agarose gel (containing 1-2.5% of formaldehyde), followed by staining with ethidium bromide or other nucleic acid dyes like "Syber Green" (Molecular Dynamics), followed by photodocumentation and by analysis after hybridizations with specific probes (Northern Blot analysis);

d) messenger RNA was reverse-transcribed generating the corresponding double-strand cDNA that was cloned into a plasmid vector, cosmid or phage vector, in order to allow its "in vitro" amplification;

e) the cDNA insert coding for the EOPA was identified and selected by hybridization, immune-selection using a specific anti-EOPA antibody, or alternatively by detection of EOPA specific activity;

f) the cDNA insert was then amplified and isolated by PCR ("polymerase chain reaction"), or alternatively by amplifying the vector containing this cDNA in bacteria, followed by digestion with restriction enzymes which allow the release of the cDNA from the vector;

g) alternatively, the amplification of the cDNA of choice was performed by direct PCR amplification, using messenger or total RNA and specific oligonucleotides (RT-PCR - reverse transcription polymerase chain reaction);

h) the cDNA was then completely sequenced, allowing the determination of the respective deduced amino acid sequence, which primary and secondary structure was analyzed, including homology search analysis using specific softwares and databanks.

Expression of the recombinant EOPA

The recombinant EOPA can be expressed using heterologous systems such as, for example, bacteria, yeast, baculovirus, eukaryotic cells in culture, and others. The production of the recombinant EOPA in bacteria was performed after subcloning the cDNA coding for the EOPA in frame into an expression vector (e.g., pProEx-HTc), such that the transcription initiation signal (including the initial methionine) of the vector was used. The *Escherichia coli* bacteria were transfected or transformed with this construction by electroporation or "heat-shock" treatment, and the expression of the respective recombinant protein was induced by the addition of IPTG (isopropyl-thio-galactosidase). The expressed protein was produced either as a recombinant or as a fusion protein, with an anchor protein or a poly-histidine tag sequence, which help in the identification and purification processes of the target protein.

The recombinant protein obtained as described above presented biochemical and immunological characteristics identical to those previously observed for the natural protein present in the brain (Hayashi et al., 2000).

The production of the recombinant EOPA, specifically in bacteria, comprised the following steps:

a) the isolated cDNA, coding for the EOPA, was cloned in frame into a plasmidial expression vector, such that the production of EOPA was achieved as a recombinant molecule or as a fusion protein with anchor proteins or a oligopeptide tag sequence;

b) the process for the production of the recombinant or fusion protein requires the transformation of the host bacteria (*Escherichia coli* strains DH5α, BL21(DE3) or similar) with the plasmidial vector construct, as described above, containing the cDNA insert coding for the EOPA cloned in frame in the expression vector; the transformation can be performed by heat-shock or electroporation of the bacteria, previously prepared using the appropriate protocol for each of these methods;

c) the transformed bacteria, obtained as described above, were amplified by growth in appropriate culture medium (usually LB medium containing appropriate antibiotics for selection), until obtaining the optic density lectures of about 0.6 at wavelengths between 560 and 600 nm; at this point, the production of the target protein was induced by addition of expression induction compounds, e.g. IPTG (isopropyl-thiogalactosidase), to a final concentration of about 0.5 to 1 mM, followed by incubation at 30°C or 37°C in a shaker;

e) when the protein was obtained as a fusion protein, it was separated from other soluble bacterial proteins (contaminants) using affinity columns, containing immobilized nickel for poly-histidine fusions or glutathione-sepharose resin for fusions with GST (glutathione-S-transferase);

subsequently, the recombinant protein was obtained by cleaving off the anchor protein or polypeptide through digestion with specific proteases (like thrombin, factor X, enterokinase, etc), whose cleavage sites had been introduced between the anchor protein and the recombinant protein of choice;

f) the purity and the quality of the recombinant protein was evaluated by electrophoresis in polyacrylamid gel (SDS/PAGE), Western Blot, mass spectrometry, and determination of the specific activity by enzymatic kinetics, using natural and synthetic peptide substrates.

Determination of the gene sequence of the human EOPA

The complete sequence of the human gene coding for EOPA was determined by the sequence analysis of the human genome entirely accessible in public data bases. The *EOPA* gene comprises 9 exons and 10 introns, and is approximately 40 kb long. This gene is located at chromosome 17p12.9, relatively close to the locus of the p53 gene (separated by approximately 0.7 cM), and on the same arm of chromosome 17 on which the gene *LIS1* is located (17p13.3). In the upstream region of the EOPA gene, including the promoter region, some transcription regulatory factors binding sites were found, such as for: AP1, cMyb, SP1, nMyc, cMyc, among others.

Analysis of the promoter region of the gene encoding for EOPA

Analysis of the promoter region of the gene coding for the rabbit EOPA identified some of the main transcriptional factors responsible for the regulation of the expression of

this enzyme. Based on the luciferase activity measured for the obtained constructions, the region between the positions -725 and -648 has at least a site recognized by a transcription factor responsible for a strong repression of the expression of EOPA in gliomas (lineage C6), probably, recognized by the product of the protooncogene *c-myb* (Thiele et al., 1988). Moreover, this study indicated that the human EOPA gene shows in its upstream region, between the position -980 and -310, recognition sites that regulate positively this gene. Besides the fact that the present work was performed only using the C6 lineage, the obtained results allowed to observe that even showing low similarity for the upstream regions, both human and rabbit EOPA gene seem to be negatively regulate in gliomas, where low EOPA-like peptidase activity have been observed (enzymatic catalytic activity measurements). On the other hand, the segment of the promoter region of the EOPA gene analyzed seem to contain a recognition site for a transcription factor that acts as an activator of the expression of this gene in the studied cell lineage, e.g., C6 cells.

The family of the *myb* genes has been described as a class of oncogenes, which regulate the transcription of gene involved in the cellular proliferation and differentiation (Ness, 1999). This family is composed by three distinct genes: A-*myb*, B-*myb*, and *c-myb* (Nomura et al., 1988; Oh et al., 1999). Between the well-known properties of *c-myb* that is noteworthy to mention that it is a survive factor that inhibits the differentiation of the myeloid and erythroid cells, allowing the blocked immature cells to continue dividing (Weston, 1998). Some groups have suggested a relationship between the *c-myb* gene and the normal function of neuronal cells (Welter et al.,

1990), and also of some tumor cells lines (Gozes et al., 1987). However, the physiological function of *c-myb* gene was not determined yet.

Generation of the anti-EOPA antibodies showing anti-catalytic activity and ability to discriminate functional similar proteins

Polyclonal antibodies were generated in rats, mice and rabbits, using the purified natural EOPA or the active recombinant EOPA, expressed in bacteria, specifically in *Escherichia coli*, for the immunization. Male Balb-C mice, 7 to 8 weeks old, and weighing between 18 and 22 g were immunized with purified natural human EOPA, or catalytically active recombinant enzyme. For each of the four immunizations, 2 µg of the purified protein or 3 µg of the recombinant protein, absorbed on aluminum hydroxide [$Al(OH)_3$] were injected intradermically, in weekly intervals. Blood samples were collected one week after the last immunization, and the serum was stored at -20°C. The antiserum titer was evaluated by ELISA, using the appropriate antigen. The anti-catalytic activity of the obtained antiserum was evaluated by HPLC or fluorimetrically, using the quenched fluorescent peptides substrates. Before use, the antiserum was pre-heated at 55°C, for 5 minutes, in order to eliminate any contaminating peptidase activity. Tests to standardize the use of the antibody showed that the incubation of the anti-EOPA antibody for 30 minutes, at 37°C, with the recombinant EOPA reached the maximum inhibitory efficiency, and that this inhibition was specific, since the incubation with other tissue oligopeptidases, such as the thimet oligopeptidase (TOP) and neurolysin (NL), did not show any

inhibitory activity. Using this antibody, it was possible to discriminate the activity of the EOPA from functionally similar, but not homologous enzymes, such as the TOP and the NL, which are also present in animal tissues and cells lineages.

Proteolytic properties and biochemical characteristics of the EOPA

Kinetic assays using natural peptide substrates like bradykinin, neurotensin and opioid peptides, composed by 9 to 13 amino acid residues, were essential for the discovery and characterization of the EOPA in our laboratory (see the summary of this literature in Camargo et al., 1994). Since 1990, we have developed peptide substrates derived from natural substrates with quenched fluorescence, standardizing the characterization of this enzyme, simplifying and reducing the costs, and making the characterization of the kinetic properties of the EOPA more reliable (Juliano et al., 1990). The peptide substrates with quenched fluorescence (qf) used have the radical Abz (*ortho*-aminobenzoic acid) at the amino terminus of the peptide, and the EDDnp [N-(2,4-dinitrophenyl) ethylenediamine] at the carboxyl terminus of the peptide. The amino acid residues of these peptides are abbreviated by the one letter code, as follow:

G-glicina	N-asparagina
A-alanina	Q-glutamina
P-prolina	D-ácido aspártico
V-valina	E-ácido glutâmico
I-isoleucina	K-lisina
L-leucina	R-arginina

S-serina	F-fenilalanina
T-treonina	H-histidina
W-triptofano	Y-tirosina

Currently, the peptide of choice used for the determination of the EOPA activity is Abz-GFAPFRQ-EDDnp. This method of characterization of the natural EOPA was applied to the recombinant enzyme, completely reproducing the results obtained with the natural EOPA (Hayashi et al., 2000). This method for enzymatic assay was largely employed to test several peptide substrates with quenched fluorescence, which was used to characterize several tissue oligopeptidases such as the EOPA, TOP and NL (Oliveira et al., 2001).

The natural protein, as well as the recombinant form, hydrolyzes peptides of 7 to 13 amino acid residues selectively, and their isoelectric point is between 5.22 and 5.50 (reviewed by Medeiros, 1992). This enzyme specifically hydrolyzes the Phe^{5}-Ser^{6} bond of bradykinin (Camargo et al., 1973), and the Arg^{8}-Arg^{9} bond of neurotensin (Camargo et al, 1983), and also releases [Met^{5}]- or [Leu^{5}]-enkephalin from several opioid peptides, which contain enkephalin in their sequences (Camargo et al., 1985; 1987), suggesting its possible involvement in the biotransformation of bioactive peptides.

The EOPA is a thiol-activated endopeptidase, insensible to the EDTA, presenting a molecular mass of approximately 40 kDa, and can be found associated to other cytosolic proteins, generating complexes with higher molecular masses. This last aspect was verified by gel filtration of the cytosol of animal tissues, specifically the cytosol of rat brain, performed using a Superose 12 column (Amersham Bioscience) calibrated with known

molecular weight standards. The proteins present in the efflux were monitored by measuring the enzymatic activity, Western blot analysis and ELISA, using the anti-EOPA antibodies.

Inhibitors of the EOPA

The state of the art has shown by kinetic assays that EOPA is a thiol-activated enzyme, since it is activated by the reducing agent dithiothreitol (DTT), and also inhibited by thiol agents like the *p*-chloromercuribenzoate (PCMB) and the 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), classic inhibitors of the cysteinyl proteases (Oliveira et al., 1976; Camargo et al., 1983; Hayashi et al., 1996 and 2000). Later, using mimetic compounds of the enzyme substrates (derivatives of the dynorphin), which were labeled with a thiol-reactive group Npys (S-(3-nitro-2-pyridine sulfenyl), the existence of a critical cysteine residue close to the catalytic site was demonstrated (Gomes et al., 1993; Hayashi et al., 1996). This critical cysteine residue of EOPA reacted irreversibly with the site-directed inhibitor, thereby corroborating with the hypothesis that this enzyme is a cysteine protease.

Therefore, EOPA is a thiol-dependent endooligopeptidase, insensible to the EDTA. However, it can be inhibited by compounds specially designed to inhibit metalloproteases, like the JA2 (Shrimpton et al., 2000), but not by the phosphinic peptide inhibitor synthesized by Jiracek and collaborators (1995). The JA2 inhibitor was obtained by modifications in the structure of the cFP, a specific inhibitor for TOP, but soon after it was verified by the inventors that both compounds inhibited the EOPA activity with a similar K_i of approximately 17 nM.

Inhibition of the endooligopeptidases by JA2 and the anti-EOPA antibody

Inhibitor / Enzyme	rEOPA	Thimet oligopeptidase	neurolysin
JA2	K_i = 17 nM	*K_i = 23 nM	K_i > 200 nM
Anti- EOPA	100% of inhibition	n.i.	n.i.

* Shrimpton et al., 2000

Distribution of EOPA in rat tissues

The expression of the EOPA in distinct rat tissues and cells was analyzed by biochemical and immunological methods, and also by employing molecular biology techniques, which means by evaluating/quantifying the specific catalytic activity, immunohistochemistry, hybridization *"in situ"* and Northern blot analysis. Using these assays, it was possible to verify a higher expression of EOPA in the brain, and show strong hybridization signals mainly in some layers of the cortex, hippocampus, cerebellum, and the basal nucleus of Meynert, indicating that the transcription level of the specific messenger RNA is in these regions was higher.

Furthermore, tissular and cellular distribution studies by immunohistochemistry has demonstrated the co-localization of EOPA with opioid peptides and their precursors in the central nervous system, and in the cellular body and neuronal axons of the retina of vertebrates, which are rich in [Leu^5]-enkephalin, as previously shown (Oliveira et al., 1990; Paik et al., 1992; Ferro et al., 1991; Hayashi et al., 2000). There are also evidences that the EOPA is secreted into the extracellular space, like other peptide messenger metabolizing enzymes.

Among the tissues (brain, testes, heart, spleen, liver, lung, skeletal muscle, and kidney) analyzed for the proteolytic activity, we observed that the cytosol of kidney showed the lowest EOPA and neurolysin activity levels. On the other hand, the thimet oligopeptidase (TOP) participates with 32% of the total activity in this tissue, when the substrate Abz-GFAPFRQ-EDDnp was used, showing an ubiquitous distribution of this enzyme. In general manner, the enzymatic activity of EOPA and thimet oligopeptidase is homogenously distributed in the cytosol of the most of tissues, with exception for the cytosol of the brain and kidney.

EOPA is preferentially found in the brain cytosol, accounting for 60% of the total oligopeptidase activity observed in this tissue. The activity of the thimet oligopeptidase was not detected in this cytosol, while 20% of the activity observed in this cytosol is due to neurolysin, and 20% due to the activity of other cytosolic enzymes, which hydrolyze this substrate.

In order to better demonstrate the characteristics identified above, which are objects of the present invention, the table above shows the distribution of EOPA, determined by the specific activity of the anti-EOPA antiserum, on the percentage of hydrolysis of the fluorescent substrate Abz-GFAPFRQ-EDDnp by the cytosolic endooligopeptidases.

Additionaly it can also be seen in figure 1, that refers to the percentage of endoologopeptidase activities with the substract Abz-GFAPFRQ-EDDnp.

Figure 2 is a schematic representation of the complete sequence of cDNA coding for human EOPA where is detached the segment used to the generation of the employed probes in the EOPA distribution study for hybridization "in situ".

Percentage of hydrolysis of the fluorescent substrate Abz-GFAPFRQ-EDDnp by the cytosolic endooligopeptidases.

Cytosol	EOPA	Metalooligopeptidases TOP e NL*	Others
Brain	60%	20%	20%
Testis	44%	47%	9%
Heart	37%	50%	13%
Spleen	30%	48%	22%
Liver	33%	62%	15%
Lung	28%	41%	31%
Skeletal muscle	52%	31%	17%
Kidney	8%	42%	50%

* The enzymatic activity of TOP and NL was deduced from the percentage of inhibition of the cytosolic peptidases by the phosphinic inibidors, Z-(L,D)-F-ψ-(PO_2CH_2)-(L,D)-A-R-M e P-F-ψ-(PO_2CH_2)-L-P-NH_2, which are specific for the TOP and NL, respectively (Jiracek et al., 1995).

Expression of EOPA in neuronal cells and melanomas

"*In vitro*" neuronal differentiation from pluripotent stem cells has been widely accepted as a model for basic studies of the molecular mechanisms involved in the initial steps of embryogenesis. Studies on the expression and function of EOPA have been performed with the P19 cells lineage, derived from murine embryonal carcinoma, as a model for "*in vitro*" neuronal differentiation process. Embryonic stem cells derived from the central layer of the blastocysts have been used to studying the control of the gene expression. They are also potential candidates as therapeutic agents to the treatment of neuronal

degenerations and for dopaminergic neuron transplants employed to the treatment of Parkinson's disease. Embryonic carcinoma cells are easily manipulated in culture, normally obtained from neoplasias of germinal tissues. They behave like stem cells, and present a gene expression pattern similar to that of the cells found in the inner layer of the epiblasts, and in culture, can originate typical endodermic, mesodermic or even ectodermic cells (Martin, 1980). The totipotent P19 cells (McBurney *et al.*, 1982), derived from murine embryonic carcinoma were used as differentiation model, and also in studies which aim is the identification of the necessary factors for their irreversible commitment to the distinct differentiation pathways. Undifferentiated P19 cells are characterized by a high proliferation rate, and both cultures with high densities of adhered or in suspension cells induce the differentiation process. This process can also be triggered by chemical factors, such as the retinoic acid or DMSO. For instance, it is known that, in the presence of 10^{-7} M retinoic acid, the P19 cells differentiate into neuroectodermic cells (Jones-Villeneuve et al., 1982).

In protocols based on preventing adhesion, cellular aggregates are maintained in suspension for five days, originating the following cellular types: on the seventh day neurons appear, and on the tenth day astrocytes and glial cells can be observed. Accordingly, variation in the nature and concentration of the chemical inducer may induce the P19 cells into multiple differentiation pathways leading to the formation of smooth or skeletal muscle cells, neurons or astrocytes (Edward and McBurney, 1983). Studies performed by the inventors, demonstrated an increased expression of the EOPA messenger RNA in the

embryonic corpuscles and in the neuronal differentiating cells, approximately on the eighth day after the treatment with retinoic acid, when the formation of neurites is observed. The aim of this work is to study the physiological role of EOPA and to determine the mechanisms of its genetic and protein modulation during the process of neuronal differentiation.

The PC12 lineage cells are derived from a rat tumor (transplantable rat pheochromocytoma) that reversibly responds to nerve growth factor (NGF), which induces to a neuronal phenotype, and poorly adheres to plastic and tend to grow forming small clusters (Greene et al., 1976). When maintened in culture under normal conditions, these cells are similar, in both biochemical and morphological aspects, to chromaphinic cells of fetal adrenal glands. They can differentiate in two ways: 1) into chromaphinic-like cells in response to glucocoticoids, and 2) into sympathic neuronal cells in response to neurotrophic factors like NGF or fibroblast growth factors (FGF). Furthermore, these cells express several bioactive peptides like the enkephalins, dynorphins and neurotensin.

These properties turn this lineage into an experimental model for the study of enzymes involved in the biotransformation of neuropeptides. We have previously demonstrated that the proteolytic activity of the EOPA was present in the cytosol of these cells, and that this activity was modulated in response to the addition of cAMP, and not in response to the treatment with FGF.

On the other hand, it is known that the treatment of these cells with FGF determines their differentiation into a neuronal phenotype, and axonal extensions can be observed. Then, the EOPA present in these cells migrates to the ends of the neurites, suggesting a possible role of

this enzyme in the formation of these cellular extensions, or in the formation of connections with other cells. Efforts have been done in order to clarify the real role of this protein in these cells.

In order to create a new biological model to study the physiological roles of EOPA, the process of differentiation of stem cells into neurons was standardized.

The embryonic stem cells (ES) are undifferentiated cells, derived from the inner cell mass of blastocysts, and are known to be pluripotent (Evans and Kaufman, 1981; Martin, 1981). Pluripotency is the remarkable capacity of ES cells to resume normal development within an organism, being able to populate different tissues including the germ line. Once cultivated under specific conditions, the stem cells can be maintained at the undifferentiated form during several cellular divisions, even after many passages. On the other hand, these cells can be induced to initiate an "in vitro" differentiation program. For instance, when cultivated in suspension, the stem cells spontaneously form cellular aggregates of differentiated cells named embryoid bodies, which are similar to a pre-implantation embryo. The morphological, immunohistochemical and molecular analysis show that several distinct embryonic lineage cells can be found inside an embryoid body, such as hematopoietic, neuronal, endothelial and muscular cells. These stem cells properties were the stimulus for the use of these cells as "in vitro" model for the early embryonic development, and for this reason is a very powerful tool to answer key questions as, for instance: what is the real role of EOPA in the process of nuclear migration in neuronal cells during the embryogenesis and, moreover, what is the importance of the catalytic activity of EOPA in this process, and how is the intracellular distribution of EOPA

in the undifferentiated cells, and in the neuronal precursor cells and mature neurons.

The undifferentiated embryonic stem cells (USP-1 lineage) were cultured in medium containing: DMEM, supplemented with 15% of bovine fetal serum, 100 mg/ml of penicillin/streptomycin and 2 mM of L-glutamine, 1% of non-essential amino acids, 10^{-4} M of monothioglicerol, and LIF (Leukemia Inhibitory Factor), in a final concentration of 1,000 units/ml. All the undifferentiated stem cells culture was maintained over a primary culture of previously irradiated murine fibroblasts (Neo Mef2). The differentiation was done after the formation of the embryoid bodies, where 750 cells were maintained in 20 µl of the culture medium drops over a Petri dish, during four days at 37°C, under 5% carbonic gas (during this period the cells form the cellular aggregates known as embryoid bodies). After the embryoid bodies formation, the cells were transferred to new dishes containing the culture medium described above, without the LIF and containing 0.1 to 0.5 µM retinoic acid (used as an agent for induction of the neuronal differentiation). The culture was then maintained with the differentiation inductor agent during four days, when the agent was removed and the cell culture was transferred to new dishes pre-treated with 0.22% gelatin and containing glass slides, where the cells were cultured for additional four days under the same conditions described above, but using neurobasal medium (a specific medium for neuronal cells culture, containing a lower percentage of bovine fetal serum). The selection of the neuronal precursor cells and the mature neurons was done by keeping the cells in the neurobasal medium. After completing the differentiation of the stem cells, the

differentiated cells were counted and photographed using a confocal microscopy.

A population of cells differentiated into neurons was obtained following the protocol described above, and the influence of the inhibition of the EOPA catalytic activity during the differentiation and maturation process of these neurons was analyzed after the treatment of these cells with the inhibitor JA2. The treatment of the neuronal precursor cells with this inhibitor led to an inhibition of the neurites formation and induced to an abnormal differentiation of the cells, which characterization is being done.

Role of the EOPA in the formation of the central nervous system during embryogenesis

The current view of the non-proteolytic function of EOPA is that it interacts with other cytosolic proteins through its "coiled-coil" structure, for instance, with Lis1 and Disc1. Lis1 is a member of the WD-40 family of proteins, which has this motif widely repeated (Neer et al., 1994), and acts as intracellular protein that maintains the functional machinery together performing several cellular functions (Hatakeyama et al., 1999; Kitagawa et al., 1999; Sidow et al., 1999). The interaction of the EOPA with Lis1 and Disc1 is essential for the formation of the central nervous system during the embryogenesis, and for vital functions such as the intracellular transport, neuritogenesis, nuclear mobility, and plasticity of the nervous tissue. Interactions anomalies cause neurological diseases as the lissencephaly and the Miller-Diecker syndrome (Reiner et al., 1993; Cardoso et al., 2002). Recently, another pathology describing EOPA participation is the schizophrenia.

Schizophrenic patients produce a truncated or mutant form of Disc1 that does not bind to EOPA, and this anomaly seems to be linked to the triggering of the disease (Ozeki et al., 2003; Taylor et al., 2003).

"In situ" hybridization studies, employing newly born rats or embryos brain slices, indicated an increase of the transcription level of the EOPA messenger RNA, mainly in the cortex of the newly born, 5 to 10 days-old embryos.

Furthermore, the inventors identified and demonstrated a high evolutionary conservation of the "coiled-coil" domain of the EOPA expressed in distinct animals, such as human, rabbit, mice and rat, in which the same protein was also named Nude-L or Nude-Like or Nude2 (Niethammer et al.,2000; Sasaki et al., 2000; Sweeney et al., 2001).

The helical structure and its ability to interact with other proteins seem to be related to the nuclear and neuronal migration, suggesting an important function for this structure in the process of cellular movement, which occurs mainly during embryogenesis.

Our results proved that the proteins homologous to EOPA, isolated from rat (Nude2, GenBank Acc. No. NM_133320) and from mice (Nude-L, GenBank Acc. No. AF323918), also show EOPA-like peptidase activity when peptide substrates previously employed for EOPA was used (Hayashi et al., 2000), besides showing a blockage of their proteolytic activity by EOPA specific inhibitors. These proteins seem to constitute a new protein family, presenting a highly conserved N-terminus domain, characterized by the presence of a "coiled-coil" structure, and a more divergent C-terminal domain, still presenting fragments of conserved sequences, even in organisms so distant in the evolutionary scale as, for instance, the *Aspergillus* [GenBank Acc. No. AF015037] (Sweeney et al., 2001; Kitagawa e cols., 2000).

The tissue-specific expression of EOPA could be observed in frog embryos through assays using the promoter region of the rabbit EOPA gene regulating the expression of a reporter protein, the GFP (Green Fluorescent Protein). First signs of transcription of the reporter gene were observed in embryos at the stage 13 to 15, and could be followed until approximately stage 40, when the embryo could not be maintained any more. In these experiments, the transcription factors, which regulate the expression of the studied gene, interact with specific sequences present in the promoter region triggering the expression of the reporter protein that indicate the tissues and the development stages in which the target protein is mainly being expressed. Therefore, these studies showed that the EOPA was mainly being expressed in the beginning of the neuronal tube formation during the neurulation, and in the neuronal ectoderm and prosencephalus. In later stages, a specific labeling of the skeletal muscles cells, which form the embryos tail, could also be observed.

These results were also confirmed by the whole mount "*in situ*" hybridization assays, performed with albino *Xenopus laevis* embryos, which confirmed the existence of EOPA homologous messenger RNAs along the anterior-posterior axis of the dorsal region of the embryos around the stage 19. Apparently, this staining includes neuronal tissues, more precisely the prosencephalon and rhombencephalon, extending to the spinal cord, and including the neural crest. Moreover, considering that the homozygous deleterious mutations of the EOPA gene are non-viable, assays were performed, in which the EOPA was super-expressed in *X. laevis* embryos, by injecting the specific messenger RNA, transcribed "*in vitro*", demonstrating that the over-expression of EOPA seems to interfere in the

normal formation of the nervous system. A smaller and misplaced eye was observed, as well as a serious malformation of the animal's brain, suggesting an abnormal lamination on the injected side.

Role of the EOPA in the antigen presenting process

It is well known that the opioids peptides are also immunomodulating agents (Blalock, 1989), and considering the presence of EOPA in cells of the immune system, it is possible to suggest a role for this enzyme in the immune system (Paik et al., 1992). These data and the fact that the size of the antigens presented by the MHC class I (Engelhard, 1994) coincides with the size of the peptides required for the binding with these endooligopeptidases (from 7 to 13 amino acid residues), lead the researchers of the present invention to investigate its possible participation in the process of self and non-self selection. The participation of the thimet oligopeptidase (TOP) in the process of MHC class I antigen presentation was also previously investigated by the inventors, since the epitopes presented by the MHC class I are oligopeptides of 7 to 13 amino acid residues, and they are also competitive inhibitors of this enzyme. Similar results have been reproduced *"in vitro"* with the recombinant EOPA, although subtle differences between the specificity of the EOPA and TOP were observed. We observed that the lowering in the linfoproliferation levels after the addition of oligopeptidase inhibitors in the antigen presenting cells is mainly due to the inhibition of EOPA, suggesting that this enzyme may be involved in the MHC class I antigen presentation system route. Since the expression of the MHC class I in the nervous tissue is related to a infectious

and degenerative processes (Piehl and Lidman, 2001), and to the plasticity of the central nervous system (Huh et al., 2000), the control of the peptidase or the chaperone activity of EOPA could explains its involvement in these processes, which evidently would be influenced by specific inhibitors of EOPA.

References:

Blalock, J.E. (1989) A molecular basis for bi-directional communication between the immune and neuroendocrine systems. *Physiol. Rev.* **69**, 1 - 32.

Camargo, A.C., Caldo, H., and Emson, P.C. (1983) Degradation of neurotensin by rabbit brain endo-oligopeptidase A and endo-oligopeptidase B (proline-endopeptidase). *Biochem. Biophys. Res. Commun.* **116**, 1151 - 1159.

Camargo, A.C., Caldo, H., and Reis, M.L. (1979) Susceptibility of a peptide derived from bradykinin to hydrolysis by brain endo-oligopeptidases and pancreatic proteinases. *J. Biol. Chem.* **254**, 5304 - 5307.

Camargo, A.C., Gomes, M.D., Reichl, A.P., Ferro, E.S., Jacchieri, S., Hirata, I.Y., and Juliano, L. (1997) Structural features that make oligopeptides susceptible substrates for hydrolysis by recombinant thimet oligopeptidase. *Biochem. J.* **324**, 517 - 522.

Camargo, A.C., Gomes, M.D., Toffoletto, O., Ribeiro, M.J., Ferro, E.S., Fernandes, B.L., Suzuki, K., Sasaki, Y., and Juliano, L. (1994) Structural requirements of bioactive peptides for interaction with endopeptidase 22.19. *Neuropeptides* **26**, 281 - 287.

Camargo, A.C., Oliveira, E.B., Toffoletto, O., Metters, K.M., and Rossier, J. (1987) Brain endo-oligopeptidase

A, a putative enkephalin converting enzyme. *J. Neurochem.* **48**, 1258 - 1263.

Camargo, A.C., Ribeiro, M.J., and Schwartz, W.N. (1985) Conversion and inactivation of opioid peptides by rabbit brain endo-oligopeptidase A. *Biochem. Biophys. Res. Commun.* **130**, 932 - 938.

Camargo, A.C., Shapanka, R., and Greene, L.J. (1973) Preparation, assay, and partial characterization of a neutral endopeptidase from rabbit brain. *Biochemistry* **12**, 1838 - 1844.

Cardoso, C., Leventer, R.J., Dowling, J.J., Ward, H.L., Chung, J., Petras, K.S., Roseberry, J.A., Weiss, A. M., Das, S., Martin, C.L., Pilz, D.T., Dobyns, W.B., and Ledbetter, D.H. (2002) Clinical and molecular basis of classical lissencephaly: Mutations in the LIS1 gene (PAFAH1B1). *Hum Mutat.***19**, 4-15.

Dauch, P., Vincent, J. P., and Checler, F. (1991) Specific inhibition of endopeptidase 24.16 by dipeptides. *Eur. J. Biochem.* **202**, 269 - 276.

Edwards, M.K.S, and McBurney, M.W. (1983) The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. *Dev. Biol.* **98**, 187 - 191.

Engelhard, V.H. (1994) Structure of peptides associated with MHC class I molecules. *Curr. Opin. Immunol.* **6**, 13 - 23.

Evans, M., and Kaufman, M. (1981) Establishment in culture of pluripotetial cells from mouse embryos. *Nature* **292**, 154 - 156.

Ferro, E.S., Hamassaki, D.E., Camargo, A.C., and Britto, L.R. (1991) Endo-oligopeptidase A, a putative enkephalin-generating enzyme, in the vertebrate retina. *J. Neurochem.* **57**, 1643 - 1649.

Ferro, E.S., Tambourgi, D.V., Gobersztejn, F., Gomes, M.D., Sucupira, M., Armelin, M.C., Kipnis, T.L., and Camargo, A.C. (1993) Secretion of a neuropeptide-metabolizing enzyme similar to endopeptidase 22.19 by glioma C6 cells. *Biochem. Biophys. Res. Commun.* **191**, 275 - 281.

Gomes, M.D., Juliano, L., Ferro, E.S., Matsueda, R., and Camargo, A.C. (1993) Dynorphin-derived peptides reveal the presence of a critical cysteine for the activity of brain endo-oligopeptidase A. *Biochem. Biophys. Res. Commun.* **197**, 501 - 507.

Gozes, I., Nakai, H., Byers,M., Avidor, R., Weinstein, Y., Shani, Y., and Shows, T.B. (1987) Sequential expression in the nervous system of c-myb and VIP genes, located in human chromossomal region 6q24. *Somat. Cell Mol. Genet.* **13**, 305 - 313.

Greene, L.A., and Tischler, A.S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**, 2424 - 2428.

Hatakeyama, S., Kitagawa, M., Nakayama, K., Shirane, M., Matsumoto, M., Hattori, Higashi, H., Nakano, H., Okumura, K., Onoe, K., Good, R.A., and Nakayama, K.I. (1999) Ubiquitin-dependent degradation of IκBα is mediated by a ubiquitin ligase Skp1/Cul1/F-box protein FWD1. *Proc. Natl. Acad. Sci. USA* **96**, 3859 - 3863.

Hayashi, M.A.F., Portaro, F.C.V., Tambourgi, D.V., Sucupira, M., Yamane, T., Fernandes, B.L., Ferro, E.S., Rebouças, N.A., and Camargo, A.C.M. (2000) Molecular and immunochemical evidences demonstrate that endooligopeptidase A is the predominant cytosolic oligopeptidase of rabbit brain. *Biochem. Biophys. Res. Commun.* **269**, 7-13.

Hayashi, M.A.F., Gomes, M.D., Rebouças, N., Fernandes, B. L., Ferro, E.S., and Camargo, A.C.M. (1996) Species specificity of thimet oligopeptidase (EC 3.4.24.15). *Biol. Chem. Hoppe-Seyler* **377**, 283-291.

Hayashi, M.A., Pires, R.S., Rebouças, N.A., Britto, L.R., and Camargo, A.C. (2001) Expression of endo-oligopeptidase A in the rat central nervous system: a non-radioactive in situ hybridization study. *Brain Res. Mol. Brain Res.* **89**, 86 - 93.

Huh, G.S., Boulanger, L.M., Du, H., Riquelme, P.A., Brotz, T.M., and Shatz, C.J. (2000) Functional requirement for class I MHC in CNS development and plasticity. *Science* **290**, 2155 - 2159.

Jacchieri, S.G., Gomes, M.D., Juliano, L., and Camargo, A.C. (1998) A comparative conformational analysis of thimet oligopeptidase (EC 3.4.24.15) substrates. *J. Pept. Res.* **51**, 452 - 429.

Jiracek, J., Yiotakis, A., Vincent, B., Lecoq, A., Nicolaou, A., Checler, F., and Dive, V. (1995) Development of highly potent and selective phosphinic peptide inhibitors of zinc endopeptidase 24-15 using combinatorial chemistry. *J. Biol. Chem.* **270**, 21701 - 21706.

Jones-Villeneuve, E.M.V., McBurney, M.W., Rogers, K.A., and Kalnins, V.I. (1982) Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol.* **94**, 253 - 262.

Juliano, L., Chagas, J.R., Hirata, I.Y., Carmona, E., Sucupira, M., Oliveira, E.S., Oliveira, E.B., and Camargo, A.C.M. (1990) A selective assay for endo-oligopeptidase A based on the cleavage of fluorogenic substrate structurally related to enkephalin. *Biochem. Biophys. Res. Commun.* **173**, 647 - 653.

Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Noriko, I., Hattori, K., Nakmichi, I., Kikuchi, A., Nakayama, K.I., and Nakayama, K. (1999) An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of β-catenin. *EMBO J.* **18**, 2401 - 2410.

Kitagawa, M., Umezu, M., Aoki, J., Koizumi, H., Arai, H., and Inoue, K. (2000) Direct association of Lis1, the lissencephaly gene product, with a mammalian homologue of a fungal nuclear distribution protein, rNUDE. *FEBS Letts.* **479**, 57 - 62.

Martin, G.R. (1980) Teratocarcinomas and mammalian embryogenesis. Science **209**, 768 - 776.

McBurney, M.W., Jones-Villeneuve, E.M.V., Edwards, M.K.S., and Anderson, P.J. (1982) Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature* **299**, 165 - 167.

Medeiros, M.S., Iazigi, N., Camargo, A.C., and Oliveira, E.B. (1992) Distribution and properties of endo-oligopeptidases A and B in the human neuroendocrine system. *J. Endocrinol.* **135**, 579 - 588.

Neer, E., Schmidt, C., Nambudripad, R., and Smith, T. (1994) The ancient regulatory-protein family of WD-repeat proteins. *Nature* **371**, 297 - 300.

Ness, S.A. (1999) Myb binding proteins: regulators and cohorts in transformation. *Oncogene* **18**, 3039 - 3046.

Niethammer, M., Smith, D.S., Ayala, R., Peng, J., Ko, J., Lee, M.S., Morabito, M., and Tsai, L.H. (2000) NUDEL is a novel Cdk5 substrate that associates with LIS1 and cytoplasmic dynein. *Neuron* **28**, 697 - 711.

Nomura, N., Takahashi, M., Matsui, M., Ishii, S., Date, T., Sasamoto, R., and Ishizaki, R. (1988) Isolation human cDNA clones of myb related genes, A-myb and B-myb. *Nucleic Acids Res.* **16**, 11075 - 11089.

Oh, I.H., and Reddy, E.P. (1999) The myb gene family in cell growth, differentiation and apoptosis. *Oncogene* **18**, 3017 - 3033.

Oliveira, E.B., Martins, A.R., and Camargo, A.C. (1976) Isolation of brain endopeptidases: influence of size and sequence of substrates structurally related to bradykinin. *Biochemistry* **15**, 1967 - 1974.

Oliveira, E.S., Leite, P.E., Spillantini, M.G., Camargo, A.C., and Hunt, S.P. (1990) Localization of endo-oligopeptidase (EC 3.4.22.19) in the rat nervous tissue. *J. Neurochem.* **55**, 1114 - 1121.

Oliveira, V., Campos, M., Melo, R.L., Ferro, E.S., Camargo, A.C., Juliano, M.A., and Juliano, L. (2001) Substrate specificity characterization of recombinant metallo oligo-peptidases thimet oligopeptidase and neurolysin. *Biochemistry* **40**, 4417 - 4425.

Ozeki, Y., Tomoda, T., Kleiderlein, J., Kamiya, A., Bord, L., Fujii, K., Okawa, M., Yamada, N., Hatten, M.E., Snyder, S.H., Ross, C.A., and Sawa, A. (2003) Disrupted-in-Schizophrenia-1 (DISC-1): mutant truncation prevents binding to NudE-like (NUDEL) and inhibits neurite outgrowth. *Proc Natl Acad Sci USA.* **100**, 289 - 294.

Paik, S.H., Betti, F., Camargo, A.C., and Oliveira, E.S. (1992) Presence of endo-oligopeptidase (EC 3.4.22.19), a putative neuropeptide-metabolizing endopeptidase in cells of the immune system. *J. Neuroimmunol.* **38**, 35 - 44.

Piehl, F., and Lidman, O. (2001) Neuroinflammation in the rat--CNS cells and their role in the regulation of immune reactions. *Immunol. Rev.* **184**, 212 - 225.

Portaro, F.C., Gomes, M.D., Cabrera, A., Fernandes, B.L., Silva, C.L., Ferro, E.S., Juliano, L., and Camargo,

A.C. (1999) Thimet oligopeptidase and the stability of MHC class I epitopes in macrophage cytosol. *Biochem. Biophys. Res. Commun.* **255**, 596 - 601.

Portaro, F.C., Hayashi, M.A., Silva, C.L., and Camargo, A.C. (2001) Free ATP inhibits thimet oligopeptidase (EC 3.4.24.15) activity, induces autophosphorylation in vitro, and controls oligopeptide degradation in macrophage. *Eur. J. Biochem.* **268**, 887 - 894.

Reiner, O., Carrozzo, R., Shen, Y., Wehnert, M., Faustinella, F., Dobyns, W.B., Caskey, C.T., and Ledbetter, D.H. (1993) Isolation of a Miller-Dieker lissencephaly gene containing G protein β-subunit-like repeats. *Nature* **364**, 717 - 721.

Rogister, B., Ben-Hur, T., and Dubois-Dalcq, M. (1999). From neural stem cells to myelinating oligodendrocytes. *Mol. Cell. Neurosci.* **14**, 287 - 300.

Sasaki, S., Shionoya, A., Ishida, M., Gambello, M. J., Yingling, J, Wynshaw-Boris, A., and Hirotsune, S. (2000) A LIS1/NUDEL/cytoplasmic dynein heavy chain complex in the developing and adult nervous system. *Neuron* **28**, 681-696.

Shrimpton, C.N., Abbenante, G., Lew, R.A., and Smith, I. (2000) Development and characterization of novel potent and stable inhibitors of endopeptidase EC 3.4.24.15. *Biochem. J.* **345**, 351 - 356.

Sidow, A., Bulotsk, M.S., Kerrebrock, A.W., Birren, B., Altshuler, D., Jaenisch, R., Johnson, K.R., and Lander, E.S. (1999) A novel member of the F-bos/WD40 gene family, encoding dactylin, is disrupted in the mouse dactylaplasia mutant. *Nature Gen.* **23**, 104 - 107.

Silva, C.L., Portaro, F.C., Bonato, V.L., Camargo, A.C., and Ferro, E.S. (1999) Thimet oligopeptidase (EC 3.4.24.15), a novel protein on the route of MHC class

I antigen presentation. *Biochem. Biophys. Res. Commun.* **255**, 591 - 595.

Sondek, J., Bohm, A., Lambright, D.G., Hamm, H.E., and Sigler, P.B. (1996) Crystal structure of a G-protein beta gamma dimer at 2.1 A resolution. *Nature* **379**, 369 - 374.

Sweeney, K.J., Clark, G.D., Prokscha, A., Dobyns, W.B., and Eichele, G. (2000) Lissencephaly associated mutations suggest a requirement of the PAFAH1b heterotrimeric complex in brain development. *Mech. Dev.* **92**, 263 - 271.

Sweeney, K.J., Prokscha, A., and Eichele, G. (2001) NudE-L, a novel Lis1-interacting protein, belongs to a family of vertabrate coiled-coil proteins. *Mech. Dev.* **101**, 21 - 33.

Taylor, M.S., Devon, R.S., Millar, J.K., and Porteous, D.J. (2003) Evolutionary constraints on the Disrupted in Schizophrenia locus. *Genomics* **81**, 67-77.

Thiele, C.J., Cohen, P.A., and Israel, M.A. (1988) Regulation of c-myb expression neuroblastoma cells during retinoic acid-induced differentiation. *Mol. Cell Biol.* **8**, 1677 - 1683.

Vincent, B., Beaudet, A., Dauch, P., Vincent, J.P., and Checler, F. (1996) Distinct properties of neuronal and astrocytic endopeptidase 3.4.24.16: a study on differentiation, subcellular distribution and secretion processes. *J. Neuroscience* **15**, 5049 - 5059.

Welter, C., Henn, W., Theisinger, B., Fischer, H., Zang, K.D., and Blin, N. (1990) The cellular myb oncogene is amplified, rearranged and activated in human glioblastoma cell lines. *Cancer Lett.* **52**, 57 - 62.

Weston, K. (1998) Myb proteins in life, death and differentiation. *Curr. Opin. Genet. Dev.* **8**, 76 - 81.

CLAIMS

What is claimed is:

Claim 1 – Process for determining the primary structure of the messenger RNA coding for the recombinant endooligopeptidase A (EOPA) and its protein sequence, in particular from human, characterized by the following steps:

a) isolating total RNA from several animal tissues, specifically human, using the guanidine isothiocyanate-phenol-chloroform extraction method or alternatively specific reagents such as the Trizol® (GibcoBRL);

b) purifying the messenger RNA from total RNA using pre-packed oligo-dT cellulose columns or the resin in suspension;

c) analyzing the quality of the obtained messenger RNA by electrophoresis in denaturing agarose gel (containing 1-2.5% of formaldehyde), followed by staining with ethidium bromide or with others nucleic acid dyes, photodocumentation, and analysis by hybridization (Northern Blot analysis);

d) reverse-transcription of the obtained messenger RNA and synthesis of the complementary DNA strand;

e) cloning of the obtained double-strand cDNA into a plasmid vector, cosmid or phage vector to allow its "in vitro" amplification;

f) identifying and selecting the EOPA cDNA by hybridization, immune-selection using a specific anti-EOPA antibody or, alternatively, by detection of EOPA specific activity;

g) amplifying and isolating the target cDNA by PCR ("polymerase chain reaction"), or alternatively by

amplifying the bacteria transformed with the vector containing this cDNA insert, in bacteria, followed by digestion with appropriate restriction enzymes in order to isolate the cDNA insert.

Claim 2 - Process of claim 1, wherein the nucleic acid dyes described in the step (c) is the "Syber Green" (Molecular Dynamics).

Claim 3 - Process of claim 1, wherein the amplification of the cDNA described in the step (f) is obtained by amplifying the bacteria transformed with the vector containing the cDNA coding for EOPA.

Claim 4 - Process of claim 1, wherein the amplification of the cDNA insert described in the step (f) is obtained alternatively by direct PCR amplification, using total or messenger RNA and specific oligonucleotides, by RT-PCR (reverse-transcription polymerase chain reaction).

Claim 5 - Process of claim 1, wherein the identification and selection of the EOPA cDNA, as described in the step (e), is performed by hybridization or, alternatively, by immune-selection using a specific anti-EOPA antibody, characterized by the following steps:

a) amplification of the cDNA library and transferring the genetic and/or protein material of each individual clone to nitrocellulose membranes by capillarity;

b) hybridization assays using the membranes obtained in the last step and probes, labeled radioactively or not, or alternatively identification of the positive clones using specific antibodies;

c) identification of the positive clones for the employed probes or antibodies, and collection of these isolated clones for later amplification and sequencing of the respective cDNA insert.

Claim 6 - Process of claim 1, wherein the fragment of about 600 base pairs, as described in the step (f), obtained by digestion of the cDNA coding for EOPA with appropriate restriction enzymes is used for the synthesis of the probes employed for the distribution analysis of the EOPA in brain by "in situ" hybridization.

Claim 7 - Process of claim 6, wherein the restriction enzymes employed are EcoRI and XbaI.

Claim 8 - Process, wherein the cDNA insert, obtained as described in the claim 1 or 2, was completely sequenced in order to determine the respective nucleotide and deduced amino acid sequence, which primary and secondary structure was also analyzed; furthermore, the determined sequence was also used in the search for homologous sequences deposited in specialized data banks.

Claim 9 - Process, as described in the claim 1 to 8, characterized by the fact that it is particularly employed for the determination of the nucleic primary structure and the respective protein from mammals.

Claim 10 - cDNA insert coding for EOPA, characterized by the fact that it comprises a fragment of about 600 base pairs.

Claim 11 - cDNA insert, as described in the claim 10, characterized by the fact that the complete sequencing of the cDNA insert allows the determination of the nucleic

primary sequence and the respective amino acid deduced sequence, which permit the analysis of the primary and secondary structure of this protein sequence.

Claim 12 - cDNA insert, as described in the claims 10 and 11, characterized by the fact that the complete sequencing of the full-length allows the analysis of the homology with other sequences deposited in specialized data banks.

Claim 13 - Use of the cDNA insert of claim 10, characterized by the fact that it was used for the synthesis of the probes employed in the distribution analysis of EOPA in brain by "in situ" hybridization.

Claim 14 - Process for the production of the human recombinant EOPA, using the cDNA isolated and identified according to the claim 1, specifically using bacteria, wherein the process comprises the steps of:

a) subcloning of the identified and isolated cDNA coding for EOPA, in frame, into the expression vector, such that EOPA is produced as a recombinant molecule or as a fusion proteins linked to anchors proteins or oligopeptides;

b) transforming the host bacteria (*Escherichia coli* strains DHα or BL21(DE3) or similar) with the plasmidial construct described above, that is, the expression vector containing the cDNA insert coding for the EOPA cloned in frame, as described in step (a);

c) amplifying the transformed bacteria by growth in appropriate culture medium, such as LB medium containing antibiotics for selection, until obtaining optic density of about 0.6 for readings at wavelengths between 560 and 600 nm;

d) production of the target protein by addition of inducing agents such as the IPTG (isopropyl-thiogalactosidase) to the final concentration of about 0.5 to 1 mM, followed by incubation at 30°C or 37°C, with shacking;

e) collecting the bacteria containing the target expressed protein by centrifugation, and storage at -20°C or immediate processing, being lyzed either by sonication or by mechanical pressure, to release the recombinant or the fusion protein present in the bacteria cytoplasm;

f) recovering and purifying the fusion protein using affinity columns, electrophoresis, or liquid-phase chromatography;

g) evaluating the purity and the quality of the recombinant protein by electrophoresis in polyacrylamide gels (SDS/PAGE), HPLC with an UV/vis detector, Western Blotting, mass spectrometry, and/or determining the specific activity, by enzymatic kinetics, using natural and/or synthetic peptide substrates.

Claim 15 - Process, as described in the claim 14, wherein the identified and isolated cDNA coding for EOPA described in the step (a) is obtained as described in the process of claim 1 to 9.

Claim 16 - Process, as described in the claim 14, wherein the subcloning described in the step (a) allows the expression of the recombinant EOPA or the EOPA in the fusion form with an anchor protein or polypeptide, which facilitate its purification.

Claim 17 - Process as described in the claim 14, characterized by the fact that in the step (b) the employed host bacteria (*Escherichia coli*) is the strain DHα or BL21(DE3) or similar.

Claim 18 - Process as described in the claim 14, characterized by the fact that in the step (c) the employed host bacteria (*Escherichia coli*) is the strain DHα or BL21(DE3) or similar.

Claim 19 - Process as described in the claim 14, characterized by the fact that in the step (c) the employed culture medium is the LB containing the antibiotic for selection.

Claim 20 - Process as described in the claim 14, wherein the induction of the expression described in the step (d) is done by addition of the inducing agent IPTG (isopropyl-thiogalactosidase).

Claim 21 - Process as described in the claim 14, characterized by the fact that in the processing of the bacteria pellet, as described in the step (e), the rupture of the bacteria is performed by sonication or mechanical pressure, in order to release the recombinant or the fusion protein present in the bacteria cytoplasm; the expressed protein can be purified by using affinity columns, electrophoresis or liquid-phase chromatography.

Claim 22 - Process as described in the claim 14, wherein the bacteria described in the step (e) are *Escherichia coli* strain DHα or BL21(DE3) or similar.

Claim 23 - Process as described in the claim 14, wherein the transformation of the host cells can be performed by

heat-shock or electroporation of the bacteria, previously and properly, prepared for each of these methods.

Claim 24 - Process as described in the claim 14, wherein the recombinant protein expressed as a fusion protein can be separated from other bacterial soluble proteins (contaminants) using affinity columns containing immobilized nickel, for poly-histidine fusions, or glutathione-sepharose resin, for fusions with GST (glutathione-S-transferase).

Claim 25 - Process as described in the claim 24, wherein the recombinant protein expressed as a fusion protein can be separated from the anchor protein or polypeptide by digestion with proteases, which consensus recognition sequences had been previously inserted between the anchor and the recombinant protein.

Claim 26 - Process as described in the claim 25, wherein the employed proteases are the thrombin, factor X, and enterokinase.

Claim 27 - Process for the determination of the EOPA gene primary structure, wherein the EOPA gene is formed by 9 exons and 10 introns, comprising about 40 kb, located at the chromosome 17p12.9, relatively close to the p53 locus (about 0.7 cM distant), at the same chromosome as the Lis1 gene (17p13.3); some recognition sites for transcriptional regulatory factors were observed in the promoter region of the gene.

Claim 28 - Process as described in the claim 27, wherein the putative binding sites for transcription regulatory factors, such as the AP1, cMyb, SP1, nMyc, cMyc, among

others, were identified in the upstream region of the EOPA gene, including the promoter region.

Claim 29 - Process as described in the claim 27, wherein the complete sequence of the human EOPA gene was determined by the analysis of the human genome complete sequence accessible at specialized data banks.

Claim 30 - Process as described in the claim 27 to 29, wherein the complete primary structure of the human EOPA gene was determined by the processes described in the claims 1 to 9.

Claim 31 - Human EOPA gene, characterized by the fact that it is formed by 9 exons and 10 introns, comprising about 40 kb, located at the chromosome 17p12.9, relatively close to the p53 locus (about 0.7 cM distant), at the same chromosome as the Lis1 gene (17p13.3); some recognition sites for transcriptional regulatory factors were observed in the promoter region of the gene.

Claim 32 - Gene as described in the claim 31, characterized by the fact that the transcription regulatory factors are AP1, cMyb, SP1, nMyc, cMyc, among others.

Claim 33 - Gene as described in the claims 31 and 32, characterized by the fact that it is related to the primary structure of human EOPA.

Claim 34 - Process for isolation, purification and determination of the amino acid sequence of the natural EOPA, wherein the method comprises the following steps:

a) homogenization of fresh tissue in 10mM Tris-HCl pH 7.5 buffer, containing 0.25M de sacarose, in the proportion of 1:3 [weight:volume];

b) ultracentrifugation of the sample at 25,000 xg for 15 minutes, and 100,000 xg for 1 hour;

c) analysis of the cytosolic fraction (supernatant) by polyacrilamide gel electrophoresis in denaturing conditions (SDS/PAGE), and protein quantification by the method of Bradford or Laemmli, followed by the Western blotting using specific antibodies;

d) fractionation of the cytosolic fraction by gel filtration chromatography using the Superose column coupled to a liquid chromatography system (Äkta - Amersham Biosciences) using the 20mM Tris-HCl pH 7.4 buffer containing 0.5M of NaCl, at a flow rate of 0.25 ml/min;

e) determination of the relative molecular weight based on a pre-calibration of the column with standard proteins dissolved in the buffer described above;

f) identification of the fraction containing the natural EOPA by determination of the specific enzymatic activity fluorimetrically, using quenched fluorescent peptides, or by using HPLC systems and natural peptide substrates;

g) purification of the natural EOPA using HPLC system coupled to a UV detector system, using the 20mM Tris-HCl pH 7.4 buffer containing 0.5M of NaCl and collecting manually the peaks corresponding to the pure EOPA, which was concentrated by liophylization and analyzed by polyacrilamide gel electrophoresis.

Claim 35 - Process as described in the claim 34, wherein the fresh tissue homogenized in the step (a) is from brain.

Claim 36 - Process as described in the claim 34, wherein the HPLC peak corresponding to the pure natural EOPA observed in the step (g) shows a single band of about 40 kDa.

Claim 37 - Process as described in the claim 34, wherein the purification of the natural EOPA may be done by using immunoaffinity columns prepared using pre-activated CH sepharose 4b resin for the covalent linkage of the N-terminus of the anti-EOPA antibody.

Claim 38 - Process as described in the claim 34, wherein the natural pure EOPA may be eluted in its active form by using 2M NaI in 50mM Tris-HCl pH 8.0 buffer, containing 20mM of NaCl.

Claim 39 - Process as described in the claim 34, wherein the EOPA primary sequence may be determined by mass spectrometry, where the data may first be acquired from the quadrupolo (ES-MS) by scanning the ratio (m/z), and the sequencing may be determined by using a second quadrupolo (ES-MS/MS) by selection of the peptides with a protonated ion characterized in the first quadrupolo, followed by a fragmentation performed by collision induced dissociation (CID).

Claim 40 - Process as described in the claim 34, wherein the mass spectrometer (ES-MS/MS) is operated in the positive ionization mode, equipped with an electrospray ion source and using software for the data acquisition.

Claim 41 - Process as described in the claim 34, wherein the sequencing may be performed by the HPLC system coupled to the mass spectrometer (LC-ESMS/MS).

Claim 42 - Process as described in the claim 34, wherein the amino-terminus of the protein may be sequenced by the Edman degradation method or deduced from amino acid composition analysis.

Claim 43 - Amino acid sequence of natural EOPA, characterized by the fact that the primary sequence may be determined by mass spectrometry, where the data may first be acquired from the quadrupolo (ES-MS) by scanning the ratio (m/z), and the sequencing may be determined by using a second quadrupolo (ES-MS/MS) by selection of the peptides with a protonated ion characterized in the first quadrupolo, followed by a fragmentation performed by collision induced dissociation (CID).

Claim 44 - Fraction containing the natural EOPA, characterized by the fact that the HPLC peak corresponding to the pure natural EOPA shows a single band of about 40 kDa.

Claim 45 - Process for the generation of the polyclonal anti-EOPA antibodies, specifically anti-human EOPA polyclonal antibodies generated in mice, which method comprises the following steps:

a) generating polyclonal antibodies by immunization with natural EOPA purified from animal brain, or using active recombinant protein produced in bacteria;

b) immunizing Balb-C or High III mice, 7 to 8 weeks old, weighing 18 to 22 g;

c) performing intradermical injections of 2 µg of the purified protein or 3 µg of the recombinant protein, absorbed on aluminum hydroxide [$Al(OH)_3$] or on

incomplete Freud's adjuvant, in weekly or monthly intervals for each of the four immunizations;

d) collecting blood samples, one week or one month after the last immunization, and the serum can be stored at -20°C;

e) evaluating the antiserum titer by ELISA and Western blot, using the appropriate antigen;

f) evaluating the anti-EOPA antiserum, using as substrate the natural peptide bradykinin or the fluorescent substrate Abz-GFAPFRQ-EDDnp, whose cleavages are monitored by HPLC and/or by fluorimetry, and verification of the anti-serum ability to block the EOPA peptidase activity.

Claim 46 - Process as described in the claim 45, wherein said the generation of the polyclonal antibodies against EOPA is specific for the anti-human EOPA raised in mice.

Claim 47 - Polyclonal antibodies anti-EOPA, characterized by the fact that they are obtained by the process described in the claims 45 and 46.

Claim 48 - Process for the characterization of the biochemical and proteolytic properties of EOPA, characterized by the fact that kinetic assays using natural peptide and/or synthetic fluorescent substrates was employed.

Claim 49 - Process, as described in the claim 48, characterized by the fact that both the natural and the recombinant protein show exactly the same enzymatic and biochemical characteristics.

Claim 50 - Process, as described in the claim 48, characterized by the fact that the EOPA is an endopeptidase thiol-activated and insensible to EDTA, showing molecular mass of approximately 40 kDa, being found associated with other cytosolic proteins generating complexes of molecular masses higher than 70 kDa.

Claim 51 - Process, as described in the claim 48, characterized by the fact that the natural EOPA and the respective recombinant protein are able to selectively hydrolyze peptides of 7 to 13 amino acid residues, they both have an isoelectric point between 5.22 and 5.50.

Claim 52 - Process described in the claim 48, wherein said both the natural and the recombinant proteins hydrolyze specifically the Phe^5-Ser^6 bond of the bradykinin, and the Arg^6-Arg^9 bond of the neurotensin, also releasing the [Met^5]- or [Leu^5]-enkephalins from several opioid peptides containing enkephalins in their sequences.

Claim 53 - Process described in the claim 48, wherein the substrates of EOPA are several neuropeptides or their derivatives, showing that it is not possible to determine the sub-sites of interaction between the substrate and the enzyme in order to predict the cleavable peptide bond, besides being demonstrated the strict correlation of the size and conformation of the substrate for the proteolytic action of the EOPA.

Claim 54 - Process for identification of EOPA inhibitors, wherein the EOPA is a thiol-dependent enzyme, since it is activated by the reducing agent dithiothreitol (DTT) and inhibited by classic inhibitors of the cysteinyl proteases, such as the thiol compounds.

Claim 55 – Process, as described in the claim 54, wherein the thiol compounds are the *p*-cloro-mercuribenzoate (PCMB) and the 5´-dithio-bis-(2-nitrobenzoic acid) (DTNB).

Claim 56 – Process, as described in the claims 54 and 55, wherein the use of the mimetic compounds of the substrates of this enzyme, like derivatives of dynorphin, labeled with a thiol-reactive group [Npys] (S-(3-nitro-2-pyridine-sulfenyl), shows the existence of a critical cysteine residue close to the catalytic site, which reacts irreversibly with the site-directed inhibitor.

Claim 57 – Process, as described in the claims 54 and 55, wherein the reactive SH group of the enzyme reacts irreversibly with the thiol-reactive group [Npys] (S-(3-nitro-2-pyridine-sulfenyl) present in the site-directed inhibitor, suggesting the enzyme is a cysteinyl protease.

Claim 58 – Process, as described in the claims 54 and 55, characterized by the fact that EOPA is a thiol-activated endooligopeptidase, insensible to the EDTA.

Claim 59 – Process as described in the claims 54 and 55, characterized by the fact that EOPA is inhibited by the metalloproteases inhibitors, such as the cFP (N-[1(R,S)-carboxy-3-phenylpropyl]) and the JA2 (N-[1(R,S)-carboxy-3-phenylpropyl]-A-α-aminoisobutiric acid-Y-p-aminebenzoate), which is a derivative synthesized from the cFP specific to the thimet oligopeptidase (TOP).

Claim 60 – Process as described in the claims 54 and 55, characterized by the fact that both compounds (JA2 and cFP) also inhibit the catalytic activity of the EOPA with a similar Ki (~17 nM).

Claim 61 - Process, as described in the claims 54 to 60, characterized by the fact that they are performed based on the process described in the claim 48.

Claim 62 - Recombinant protein, characterized by the fact that it is an endopeptidase thiol-activated and insensible to EDTA, showing molecular mass of approximately 40 kDa, being found associated with other cytosolic proteins generating complexes of molecular masses higher than 70 kDa.

Claim 63 - Protein, as described in the claim 62, characterized by the fact that it is able to selectively hydrolyze peptides of 7 to 13 amino acid residues, they both have an isoelectric point between 5.22 and 5.50.

Claim 64 - Protein, as described in the claims 62 and 63, characterized by the fact that it hydrolyzes specifically the Phe^5-Ser^6 bond of the bradykinin, and the Arg^8-Arg^9 bond of the neurotensin, also releasing the [Met^5]- or [Leu^5]-enkephalins from several opioid peptides containing enkephalins in their sequences.

Claim 65 - Protein, as described in the claims 62 to 64, characterized by the fact that it is a thiol-dependent enzyme, since it is activated by the reducing agent dithiothreitol (DTT) and inhibited by classic inhibitors of the cysteinyl proteases, such as the thiol compounds.

Claim 66 - Protein, as described in the claims 62 to 65, characterized by the fact that it shows a critical cysteine residue close to the catalytic site.

Claim 67 - Process for the utilization of the polyclonal antibodies against EOPA, wherein the anti-EOPA antibodies

are specific inhibitors of the EOPA catalytic activity (do not inhibit other peptidases, specifically TOP and NL) and interfere in the process of interaction with oligopeptides and proteins.

Claim 68 - Process as described in the claim 67, characterized by the fact that it refers to the anti-human EOPA antibody raised in mice, as described in the claim 45.

Claim 69 - Process for the utilization of the polyclonal antibodies against EOPA, wherein the distribution of the EOPA may be determined by quantifying the specific proteolytic activity, immunohistochemistry, "in situ" hybridization and/or Northern blot.

Claim 70 - Process as described in the claim 69, characterized by the fact that it refers to the anti-human EOPA antibody raised in mice, as described in the claim 45.

Claim 71 - Process as described in the claim 69, characterized by the fact that between the analyzed tissues by quantifying the specific proteolytic activity, the kidney cytosol shows the lowest oligopeptidase activity of EOPA; while the thimet oligopeptidase (TOP) is responsible of 60% of the total oligopeptidase activity of this tissue when the substrate Abz-GFAPFRQ-EDDnp is used.

Claim 72 - Process as described in the claim 71, characterized by the fact that the analyzed tissues are brain, testes, heart, spleen, liver, lung, skeletal muscle and kidney.

Claim 73 - Process as described in the claim 69, characterized by the fact that the EOPA and TOP (thimet oligopeptidase) activity being homogeneously distributed in

the cytosol of the analyzed tissues in general manner, except for the brain and kidney.

Claim 74 - Process as described in the claim 69, characterized by the fact that the EOPA has preferential distribution in the cytosol of the rat brain, presenting 60% of the total oligopeptidase activity.

Claim 75 - Process as described in the claim 74, characterized by the fact that the thimet oligopeptidase (TOP) activity was not detected in the cytosol of the rat brain.

Claim 76 - Process as described in the claim 74, characterized by the fact that it was shown that besides EOPA, 20% of the activity of this cytosol is due to the activity of NL, and other 20% are due to the action of other(s) non-identified cytosolic enzymes, which hydrolyzes the substrate.

Claim 77 - Process as described in the claim 69, characterized by the fact that the distribution of EOPA was also determined by "in situ" hybridization assays, showing that the expression of EOPA is stronger in the brain.

Claim 78 - Process as described in the claim 69, characterized by the fact that the expression of EOPA is stronger in the brain, showing higher expression level in some layers of the cortex, hippocampus, cerebellum, and the basal nucleus of Meynert.

Claim 79 - Process as described in the claim 69, characterized by the fact that the immunohistochemical studies of tissue and cellular distribution, the co-localization of the EOPA with opioid peptides and their

precursors is identified in the central nervous system, in the cell body and neuronal axons of the vertebrate retina, which are rich in [Leu^5]-encephalin.

Claim 80 – Process as described in the claim 69, characterized by the fact that the EOPA is secreted into the extracellular space, in an analogous form to other peptide messenger metabolizing enzymes.

Claim 81 – Process as described in the claim 69, characterized by the fact that the "*in situ*" hybridization studies, employing brain slices of newly born rats or embryos, suggest an increase in transcription level of the messenger RNA coding for the EOPA, mainly in the cortex of newly born embryos approximately between days 5 and 10.

Claim 82 – Process for identification of congenital, infectious and degenerative pathologic conditions of the central nervous system, characterized by the fact that the EOPA has an important role in the process of the central nervous system formation during the embryogenesis.

Claim 83 – Process as described in the claim 82, characterized by the fact that the identified EOPA presents high evolutionary conservation of the "coiled-coil" domain, and is expressed in different animals, as in human, rabbit, mice and rat.

Claim 84 – Process as described in the claim 83, characterized by the fact that the identified EOPA is expressed in different animals, and is also called as Nude-L or Nude2.

Claim 85 – Process as described in the claim 83, characterized by the fact that the "coiled-coil" domain of

the EOPA presents a helical structure, and is related to the nuclear and neuronal migration, and has an important function in the process of cellular movement occurring during embryogenesis.

Claim 86 - Process as described in the claim 84, characterized by the fact that the proteins homologous to the EOPA, isolated from rat (Nude2, GenBank Acc. No. NM_133320) and from mice (Nude-L, GenBank Acc. No. AF323918), also present oligopeptidase activity upon substrate used to characterize the EOPA, besides showing blockage of their proteolytic activity by specific inhibitors of the human, the rabbit and the rat EOPA.

Claim 87 - Process for the determination of the distribution and the role of EOPA in the cultured cells, characterized by the fact that the detection and the determination of the role of the EOPA in cell cultures is performed by assays, which identify the increased expression of the messenger RNA of the EOPA in embryonic corpuscles and in the differentiating cells, around the 8th day after the treatment with retinoic acid, when neurites are formed, and utilize the totipotent p19 cells, derived from a murine embryonal carcinoma, as a model of *"in vitro"* differentiation and for the identification of the factors regulating the differentiation process triggering.

Claim 88 - Process as described in the claim 87, characterized by the fact that the EOPA expression may be modulated at genetic and protein levels during the neuronal differentiation process.

Claim 89 - Process, as described in the claim 87, characterized by the fact that the EOPA proteolytic

activity is mainly found in the cytosol of the PC12 cells, derived from a rat tumor (transpantable rat pheochromocytoma), which oligopeptidase activity is modulated by the cAMP and/or by the treatment with compounds such as the FGF or retinoic acid.

Claim 90 – Process as described in the claim 87, characterized by the fact that the treatment of the PC12 cells with the FGF leads these cells to a differentiation to a neuronal phenotype, showing the formation of axonal outgrowth and the migration of the EOPA localization from the perinucleus cytosol to the neurites extremities, suggesting a role for EOPA in the process of cellular extensions and inter-cellular connections formation.

Claim 91 – Process for determining the role of EOPA in the immunological processes, characterized by the fact that the size of the epitopes associated with the MHC class I is similar to that required to the peptide substrates/inhibitors to be susceptible to binding to EOPA (e.g., 7 to 13 amino acid residues).

Claim 92 – Process as described in the claim 91, characterized by the fact that the epitopes behave as competitive inhibitors of this enzyme, and/or as ligands which modulate the association of the EOPA with other cellular proteins; the opioid peptides and other bioactive peptides, presenting affinity to the EOPA might act in the same way, in the central nervous system as well as in the immune system, since the EOPA is also found in cells of the immune system, in particular, in macrophages and lymphocytes.

Claim 93 - Process as described in the claim 91, characterized by the fact that the expression of the MHC class I in nervous tissue is related to infectious and degenerative processes, and with the plasticity of the central nervous system; the control of the peptidase and the chaperon activities, or of the peptide receptor activity of EOPA, is directly related to the specific ligands (substrates, inhibitors and modulators) of the EOPA.

Claim 94 - Process for the development of diagnosis methods, prevention or treatment of congenital, infectious and degenerative diseases of the central nervous system (CNS), wherein employing the sequence of the EOPA or its segments in the diagnostic methods for prevention and treatment of these pathologies.

Claim 95 - Process as described in the claim 94, wherein based on the method for the identification of EOPA as described in the claim 61.

Claim 96 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological, psychiatric, and behavioral pathologies, and for the processes of tissue degeneration, characterized by the fact that specific ligands of the EOPA are employed in the prevention and the treatment of these diseases.

Claim 97 - Process as described in the claim 96, wherein based on the process for the identification of inhibitors as described in the claims 54 and 55.

Claim 98 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological, psychiatric, and behavioral pathologies, and for the

processes of tissue degeneration, characterized by the fact that the inhibitors, antibodies and competitors compounds that interfere with the proteolytic, chaperon and/or soluble-receptor activities of the EOPA, may be used for diagnosis, prevention and treatment of these diseases.

Claim 99 - Process as described in the claim 98, wherein based on the process for the identification of inhibitors as described in the claims 54 and 55, and based on the process of the development of diagnosis methods, prevention or treatment of congenital, infectious and degenerative diseases as described in the claim 94.

Claim 100 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological diseases, and for the processes of tissue degeneration, characterized by the fact that the development of the EOPA inhibitors may be done by using the structural models based on natural toxins or compounds in general.

Claim 101 - Process as described in the claim 100, characterized by the fact that they are specially found in the animal venoms.

Claim 102 - Process as described in the claims 100 and 101, wherein based on the process for identification of EOPA inhibitors as described in the claims 54 and 55, and on the process for the development of diagnosis methods, prevention or treatment of congenital, infectious and degenerative diseases as described in the claim 94.

Claim 103 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological diseases, and for the processes of tissue degeneration, characterized by the fact that these compounds may be

employed in formulations or drugs which interfere in the process of neuropeptides inactivation or biotransformation by EOPA, besides of being used for the diagnosis, prevention and/or treatment of these diseases.

Claim 104 - Process, as described in the claim 103, wherein based on the process for the identification of inhibitors as described in the claims 54 and 55, and on the process for the development of diagnosis methods, prevention or treatment of congenital, infectious and degenerative diseases as described in the claim 94.

Claim 105 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological diseases, and for the processes of tissue degeneration, characterized by the fact that the peptide inhibitors which dislocate others peptides from the EOPA active-site may be employed.

Claim 106 - Process as described in the claim 105, wherein based on the process for the identification of inhibitors as described in the claims 54 and 55, and on the process for the development of diagnosis methods, prevention or treatment of congenital, infectious and degenerative diseases as described in the claim 94.

Claim 107 - Process for the use of inhibitors, competitors and their derivatives for the treatment of neurological diseases, and for the processes of tissue degeneration, characterized by the fact that it is possible to modulate the proteolytic/chaperona acvity of EOPA by using the inhibitors which interfere in the process of linfoproliferation or any other role in which the MHC class

I might be involved such as the infeccious, degenerative and plasticity of the central nervous system.

Claim 108 - Process as described in the claim 107, wherein based on the process for the identification of inhibitors as described in the claims 54 and 55, and on the process for the development of diagnosis methods, prevention or treatment of congenital, infectious and degenerative diseases as described in the claim 94.

Claim 109 - Process for immunochemical and/or enzymatic diagnosis, for the prevention and for monitoring the evolution of pathologies, for the prognosis and/or treatment of congenital, infectious and degenerative pathologic conditions of the central nervous system, characterized by the fact that the proteolytic activity and the interactions with other molecules properties of EOPA is regulated by inhibitors and/or substrates, or by other molecules which bind to EOPA, including the immunoglobulins.

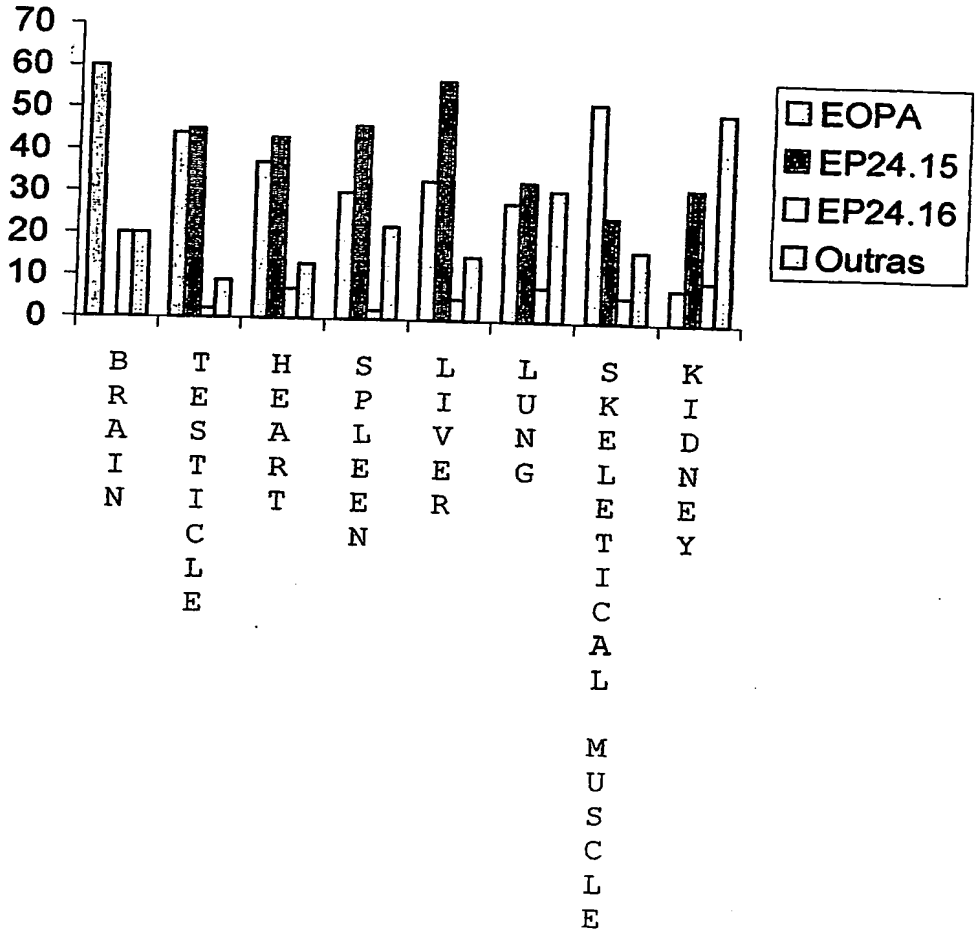
Claim 110 - Process as described in the claim 109, characterized by the fact that the peptidase activity of EOPA can be determined by kinetic assays employing natural and synthetic peptide substrates.

Claim 111 - Process for the treatment of neurological, psychiatric and neurodegenerative pathologies, characterized by the fact that EOPA is related with the process of nuclear and neuronal migration, and also formation of the central nervous system, which occur during the embryogenesis.

Claim 112 - Process as described in the claim 111, characterized by the fact that the tissular and cellular

distribution of EOPA by immunohistochemistry shows the co-localization of EOPA and the opioid peptides and their precursors in the central nervous system, more precisely, in the cellular body and axons of the neurons.

Claim 113 – Process, as described in the claim 111, characterized by the fact that the EOPA distribution can be determined by using the "in situ" hybridization assays, which allowed observing a higher expression of EOPA in the brain.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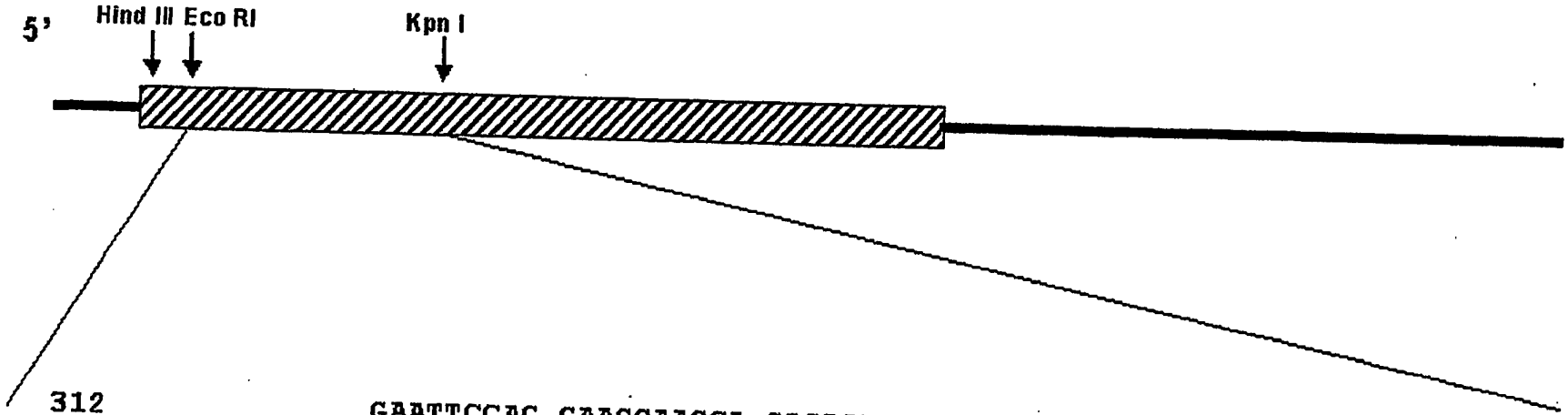
60
50
40
30
20
10
0
EOPA
EP24.15
EP24.16
Outras
BRAIN
TESTICLE
HEART
SPLEEN
LIVER
LUNG
SKELETICAL MUSCLE
KIDNEY

FIGURE 1

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481  CAGTGTTAGA AGATGATTTA AGTCAGACTC GGGCCATTAA GGAGCAGTTG CATAAGTATG
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781  CTCCTAGCTC TCCAACTCTA GACTGTGAAA AGATGGACTC CGCCGTACAA GCATCACTTT
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FIGURE 2